

# Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/96630/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Garfield, Alastair S., Davies, Jennifer S., Burke, Luke K., Furby, Hannah  
ORCID: <https://orcid.org/0000-0002-7279-1812>, Wilkinson, Lawrence ORCID:  
<https://orcid.org/0000-0002-9337-6124>, Heisler, Lora K. and Isles, Anthony  
Roger ORCID: <https://orcid.org/0000-0002-7587-5712> 2016. Increased  
alternate splicing of Htr2c in a mouse model for Prader-Willi syndrome leads  
disruption of 5HT2C receptor mediated appetite. Molecular Brain 9 , 95.  
10.1186/s13041-016-0277-4 file

Publishers page: <https://doi.org/10.1186/s13041-016-0277-4>  
<<https://doi.org/10.1186/s13041-016-0277-4>>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies.

See

<http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



RESEARCH

Open Access



# Increased alternate splicing of *Htr2c* in a mouse model for Prader-Willi syndrome leads disruption of 5HT<sub>2C</sub> receptor mediated appetite

Alastair S. Garfield<sup>1,5</sup>, Jennifer R. Davies<sup>2</sup>, Luke K. Burke<sup>3</sup>, Hannah V. Furby<sup>2</sup>, Lawrence S. Wilkinson<sup>2</sup>, Lora K. Heisler<sup>4</sup> and Anthony R. Isles<sup>2\*</sup>

## Abstract

Alternate splicing of serotonin (5-hydroxytryptamine; 5-HT) 2C receptor (5-HT<sub>2C</sub>R) pre-RNA is negatively regulated by the small nucleolar RNA, *Snord115*, loss of which is observed in nearly all individuals with Prader-Willi Syndrome (PWS), a multigenic disorder characterised by hyperphagia and obesity. Given the role of the 5-HT<sub>2C</sub>R in the regulation of ingestive behaviour we investigated the pathophysiological implications of *Snord115* deficiency on 5-HT<sub>2C</sub>R regulated appetite in a genotypically relevant PWS mouse model (PWS-IC). Specifically, we demonstrate that loss of *Snord115* expression is associated with increased levels of hypothalamic truncated 5-HT<sub>2C</sub>R pre-mRNA. The 5-HT<sub>2C</sub>R promotes appetite suppression via engagement of the central melanocortin system. *Pro-opiomelanocortin* (*Pomc*) mRNA levels within the arcuate nucleus of the hypothalamus (ARC) were reduced in PWS-IC mice. We then went on to assess the functional consequences of these molecular changes, demonstrating that PWS-IC mice are unresponsive to an anorectic doses of a 5-HT<sub>2C</sub>R agonist and that this is associated with attenuated activation of POMC neurons within the ARC. These data provide new insight into the significance of *Htr2c* pre-mRNA processing to the physiological regulation of appetite and potentially the pathological manifestation of hyperphagia in PWS. Furthermore, these findings have translational relevance for individuals with PWS who may seek to control appetite with another 5-HT<sub>2C</sub>R agonist, the new obesity treatment lorcaserin.

**Keywords:** *Snord115*, Prader-Willi syndrome, Serotonin 2C receptor, Alternate splicing, Feeding

## Introduction

Manipulations of the central serotonin (5-hydroxytryptamine; 5-HT) system elicit profound effects on feeding behaviour [1]. Specifically, a reduction in serotonin availability or efficacy, causes hyperphagia and resultant weight gain, whilst augmented serotonin bioavailability or receptor-specific agonism leads to hypophagia and weight loss. Although the anorectic action of serotonin occurs via activation of multiple receptor subtypes, it is the 5-HT<sub>2C</sub> receptor (5-HT<sub>2C</sub>R) that is the most predominant [2]. Genetic ablation of the 5-HT<sub>2C</sub>R gene

(*Htr2c*) in mice leads to hyperphagia [3], whilst receptor-specific agonists suppress food intake by enhancing the onset of satiety [4]. More recent investigation has identified the central melanocortin system as the principle mediator of 5-HT<sub>2C</sub>R regulated appetite [4–6]. The arcuate nucleus of the hypothalamus (ARC) contains two discrete populations of melanocortin neurons, those synthesising the anorectic melanocortin receptor (MCR) agonist pro-opiomelanocortin (POMC) and those synthesising the orexigenic MCR antagonist/inverse agonist agouti-related peptide (AgRP); 5-HT<sub>2C</sub>Rs are expressed on, and modulating firing of, ARC POMC neurons [7]. Furthermore, 5HT<sub>2C</sub>R expression specifically on POMC neurons is both necessary and sufficient for the promotion of satiety and the regulation of body weight [5, 8].

\* Correspondence: IslesAR1@cardiff.ac.uk

<sup>2</sup>Behavioural Genetics Group, MRC Centre for Neuropsychiatric Genetics and Genomics, Neuroscience and Mental Health Research Institute, Schools of Medicine and Psychology, Cardiff University, Cardiff, UK  
Full list of author information is available at the end of the article



© The Author(s). 2016 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated.

5-HT<sub>2C</sub>R function is influenced by two post-transcriptional processes, with the *Htr2c* pre-mRNA being subject to alternate splicing [9] and adenosine-to-inosine RNA-editing [10]. Both these events promote the translation of less functional receptors due to their effects on the amino acid sequence of the critical G-protein binding domain. Specifically, RNA-editing within exon V can result in the combinatorial conversion of five clustered adenosine residues into inosines, leading to a change in codon-specificity and subsequent amino acid sequence. Alternate splicing of exon V results in a truncated protein lacking a functional G-protein binding domain. However, although this truncated splice variant cannot act as a receptor it plays a critical role in overall 5-HT<sub>2C</sub>R function by forming a heterodimer and sequestering the full-length splice variant in the endoplasmic reticulum and reducing cell surface expression [11]. This role for the truncated 5-HT<sub>2C</sub>R has recently been confirmed and its functional importance demonstrated by microinjection into the brain of an oligonucleotide that promotes the production of the full-length transcript and, in turn, produces a change in feeding behaviour [12].

Processing of *Htr2c* pre-RNA is mediated, in part, by the actions of the small nucleolar RNA (snoRNA) *Snord115* (previously *h/mbii-52*) [13, 14] present within the imprinted Prader-Willi syndrome (PWS) locus [15]. Loss of expression of the genes in this locus gives rise to PWS, a congenital neuroendocrine disorder in which hyperphagia and obesity are the hallmark symptoms [16]. A number of *in silico* and *in vitro* studies have demonstrated that this C/D box containing snoRNA primarily regulates alternate splicing [17], in particular the processing of *Htr2c* pre-mRNA, promoting the inclusion of exon Vb and reducing the amount of RNA encoding the truncated receptor (Fig. 1) [13]. Nevertheless, although increases in RNA-editing of the *HTR2C* pre-RNA has

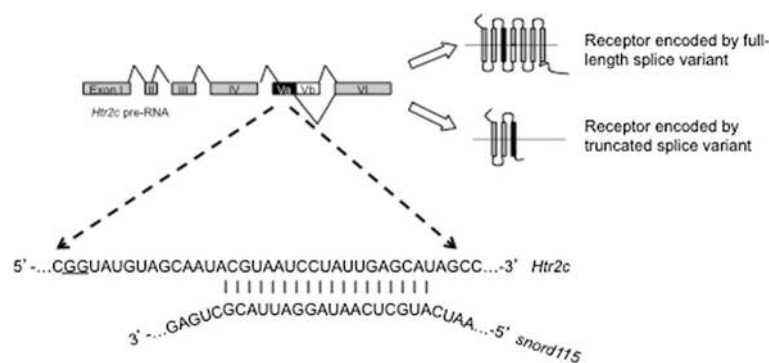
been shown in both PWS [13] and PWS mouse model brain samples [18], to our knowledge there has never been a clear demonstration of changes in alternate splicing.

Given the importance of 5-HT<sub>2C</sub>R function to the regulation of food intake, it has been suggested that loss of *SNORD115* expression may influence 5-HT<sub>2C</sub>R regulated appetite and could thus contribute to hyperphagia in PWS [13]. In this work, we examine the proportion of full-length and truncated splice variants of *Htr2c* mRNA in the PWS-IC mouse. The PWS-IC mouse is a full genetic model for PWS in which all paternally expressed genes in the cluster are silenced and in which we have previously demonstrated hyperphagia and abnormal feeding behaviour [18, 19]. We go on to assess the pathophysiological consequences of increased levels of truncated variant *Htr2c* on hypothalamic 5-HT<sub>2C</sub>R function, establishing an important role in the modulation of serotonin regulated appetite, and identify serotonin-melanocortin axis dysfunction as a potential contributing factor to hyperphagia in the clinically salient PWS-IC mouse.

## Results

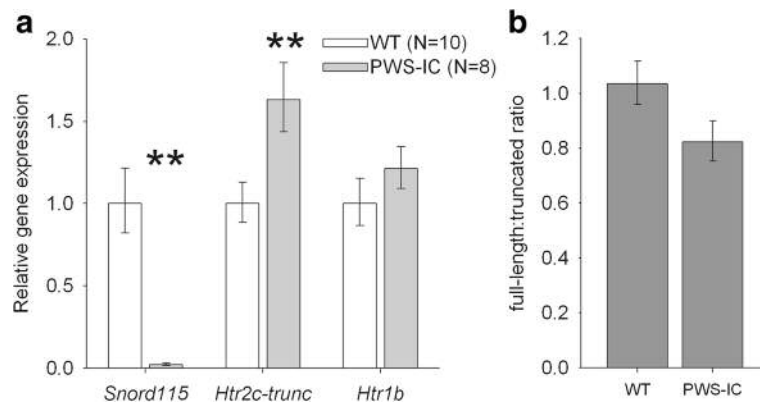
### Increased truncated *Htr2c* splice variant in hypothalamus of PWS-IC mice

Previous investigation of *Htr2c* pre-mRNA modification in PWS-IC mice at the whole brain level demonstrated an increase of RNA-editing in the absence of a significant effect on the presence of the truncated *Htr2c* mRNA isoform [18]. A more neuroanatomically refined analysis now reveals that virtually undetectable *Snord115* expression in the hypothalamus PWS-IC mice (Fig. 2a;  $t_{16} = 6.3$ ,  $p = 0.001$ ) is associated with a marked increase in levels of truncated *Htr2c* mRNA in the same samples (Fig. 2a;  $t_{16} = 3.1$ ,  $p = 0.008$ ). There was no significant difference in expression of 5-HT<sub>1B</sub>R (*Htr1b*) transcript (Fig. 2a;  $t_{16} = 0.8$ ,  $P = 0.41$ ). As expected, the increased



**Fig. 1** Schematic outlining the binding of *Snord115* to *Htr2c* and how alternate splicing can lead to full-length and truncated 5HT<sub>2C</sub>Rs. Binding of *Snord115* to a specific sequence in exon Va of the *Htr2c* pre-mRNA promotes the inclusion of exon Vb and the production of the full-length 5HT<sub>2C</sub>R; the exon/alternative exon border in the proximal splice site (GG) is underlined. Skipping of exon Vb leads to the introduction of a premature “stop” codon and the production of a truncated 5HT<sub>2C</sub>R isoform. Loss of *snord115* expression, as is expected in the majority of cases of PWS, is expected to lead to an increase in levels of the truncated 5HT<sub>2C</sub>R isoform





**Fig. 2** Increased levels of truncated *Htr2c* in PWS-IC hypothalamus. Gene expression and splice variant ratio was assessed using quantitative PCR (qPCR) in macro-dissected hypothalamus. **a** Expression of *Snord115* was absent in PWS-IC hypothalamus. This deficiency promotes alternate splicing of *Htr2c*, with levels of the truncated, non-functional, receptor isoform increased in PWS-IC mice relative to WT. The level of another regionally relevant serotonin receptor, *Htr1b* was unaltered in PWS-IC mice. **b** The consequence of increased levels of truncated *Htr2c* expression in PWS-IC hypothalamus, is a significant shift in the ratio of full-length:truncated, with a decreased proportion of functional (full-length) variants. Data presented as Mean  $\pm$  SEM. Student's *t*-test, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to WT controls

levels of truncated *Htr2c* mRNA in turn resulted in a significant decrease in the ratio of full-length:truncated *Htr2c* in PWS-IC hypothalamus (Fig. 2b; one-tailed *t*-test,  $t_{16} = 2.17$ ,  $P < 0.023$ ).

#### Altered hypothalamic neurochemistry in PWS-IC mice

In light of this, we undertook an assessment of hypothalamic neurochemistry in PWS-IC mice. Serotonin suppresses appetite, a function mediated predominantly via 5-HT<sub>2C</sub>R of the ARC melanocortin system [2, 4–6]. Analysis of serotonin regulated appetite-related neuropeptide levels by in situ hybridisation histology (ISHH) and autoradiograph densitometry revealed a significant reduction in ARC *Pomc* mRNA expression (Fig. 3;  $t_6 = 3.9$ ,  $p = 0.008$ ) but no change in that of agouti-related peptide (*Agrp*;  $t_6 = 0.4$ ,  $p = 0.681$ ) or neuropeptides Y (*Npy*,  $t_4 = 0.6$ ,  $p = 0.14$ ) mRNA expression in PWS-IC ARC, as compared to WT controls. Levels of brain-derived neurotrophic factor (*Bdnf*) mRNA within the ventromedial hypothalamus also did not vary between PWS-IC and WT mice (Fig. 3;  $t_4 = 0.6$ ,  $p = 0.58$ ).

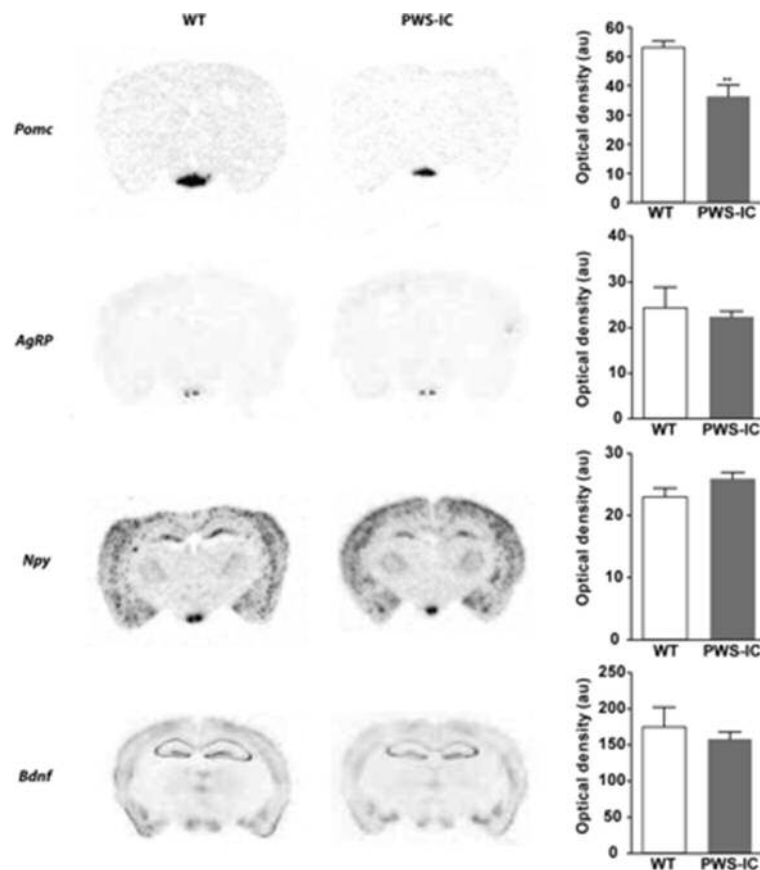
#### Blunted anorectic effect of 5-HT<sub>2C</sub>R agonist in PWS-IC mice

To directly assess the functional significance of increased levels of truncated *Htr2c* on 5-HT<sub>2C</sub>R regulated appetite we probed the response of PWS-IC mice to an anorectic dose of a 5-HT<sub>2C</sub>R specific agonist, WAY-161503 [20], in a post-fast re-feeding paradigm. Dosing with WAY-161503 produced a 20–25% decrease in food intake as expected (Fig. 4; repeated measures ANOVA, main effect of DOSE,  $F_{2,48} = 20.34$ ,  $p = 0.001$ ). As observed previously [19], PWS-IC mice consistently consumed more food than WT controls (Fig. 4; repeated measures ANOVA, main effect of GENOTYPE,  $F_{1,24} =$

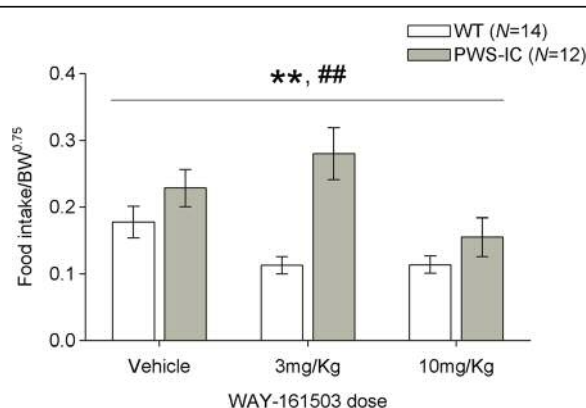
11.387,  $p = 0.003$ ) and WT and PWS-IC mice responded differently to WAY-161503 (GENOTYPE X DOSE interaction,  $F_{2,48} = 5.68$ ,  $p = 0.006$ ). *Post hoc* (Tukey's) analysis revealed that 3 mg/kg ( $p = 0.030$ ) and 10 mg/kg ( $p = 0.033$ ) WAY-161503 significantly reduced consumption in WT mice compared to vehicle treatment. However, no dose of WAY-161503 significantly reduced consumption relative to vehicle in PWS-IC mice (3 mg/Kg,  $p = 0.50$ ; 10 mg/Kg,  $p = 0.26$ ).

#### 5-HT<sub>2C</sub>R agonist induced hypothalamic neuronal activation in PWS-IC mice

The appetite suppressing actions of 5-HT<sub>2C</sub>R agonists are fundamentally associated with the activation of neurons of the ARC [4, 5, 7]. As expected, 3 mg/kg WAY-161503 significantly increased the overall number of cFOS-immunoreactive (IR) cells within the ARC (Fig. 5; two-way ANOVA, main effect of DOSE,  $F_{1,15} = 7.13$ ,  $P = 0.020$ ). However, there was also an interaction between DOSE and GENOTYPE (two-way ANOVA,  $F_{1,15} = 9.76$ ,  $P = 0.009$ ). Analysis of cell counts in WT brain, at two separate neuroanatomical levels of the ARC (−1.46 and −1.70 mm from bregma), as well as across the rostral-caudal extent of the nucleus, revealed a robust and significant increase in cFOS induction (Fig. 5a, c, e; bregma −1.46,  $t_5 = 5.3$ ,  $p = 0.010$ ; bregma −1.70,  $t_6 = 2.6$ ,  $p = 0.042$ ; Av,  $t_6 = 4.0$ ,  $p = 0.007$ ). In contrast, WAY-161503 administration failed to induce cFOS-IR at any level of the ARC in PWS-IC mice (Fig. 5b, d, f; bregma −1.46,  $t_6 = 0.3$ ,  $p = 0.75$ ; bregma −1.70,  $t_6 = 0.7$ ,  $p = 0.487$ ; Av,  $t_6 = 0.8$ ,  $p = 0.87$ ). Further investigation revealed the reduced anorectic efficacy of WAY-161503 in PWS-IC mice to be associated with a failure to induce appropriate



**Fig. 3** Perturbed hypothalamic neurochemistry in PWS-IC mice. In situ hybridisation analysis of ARC *Pomc*, *AgRP*, *Npy* and *Bdnf* mRNA expression in PWS-IC mice demonstrates a significant decrease in ARC *Pomc* mRNA expression, but no effect on ARC *AgRP*, *Npy* and *Bdnf* mRNA levels. Data presented as Mean ± SEM. Student's *t*-test, \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 compared to WT controls



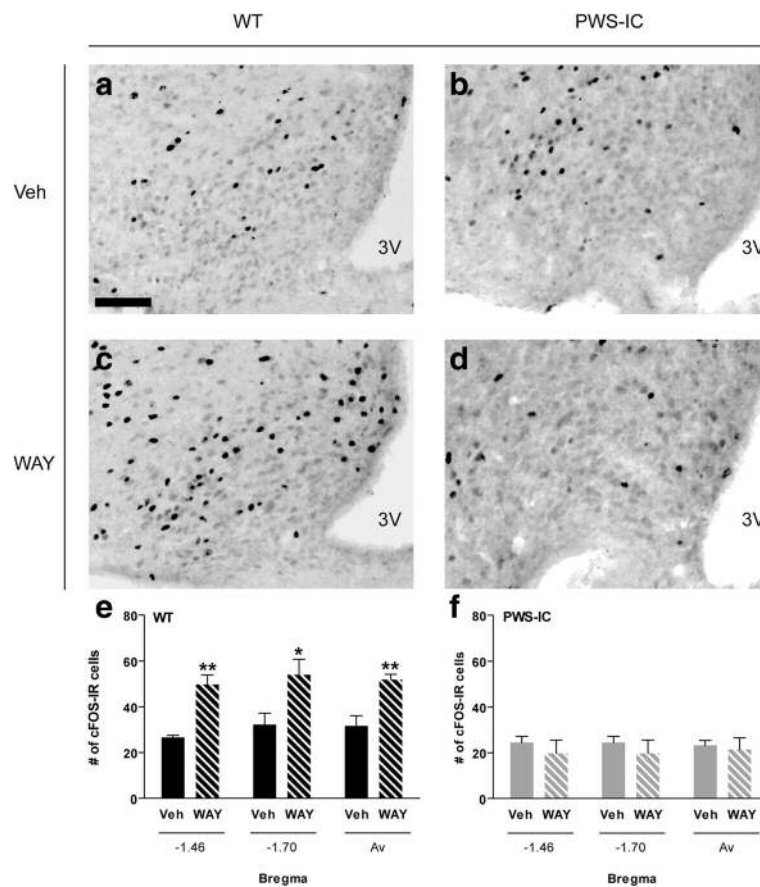
**Fig. 4** Blunted feeding effects of WAY-161503 in PWS-IC mice. 60 min food consumption upon administration of 3 mg/kg and 10 mg/kg WAY-161503 (s.c) in WT and PWS-IC mice. PWS-IC mice are unresponsive to an anorectic dose of the 5-HT<sub>2C</sub>R agonist WAY-161503. Data presented as Mean ± SEM, with statistical comparison performed by One-way repeated measure ANOVA, \**p* < 0.05 compared to vehicle controls

downstream POMC signalling as administration of 3 mg/kg WAY-161503 substantially increased the number of cFOS-IR POMC neurons within the ARC of WT (Fig. 6a, c, C'), but not PWS-IC mice (Fig. 6b, d, D').

## Discussion

The contribution of the 5-HT<sub>2C</sub>R to the central regulation of energy balance, together with the established role of *Snord115* in modulating 5-HT<sub>2C</sub>R function and lack of *SNORD115* expression in nearly all cases of PWS raises the possibility that abnormalities in 5-HT<sub>2C</sub>R-mediated feeding circuitries in brain may contribute to the overeating seen in PWS subjects. Using a model for PWS that lacks *Snord115* expression, the PWS-IC mouse, we provide evidence for this idea, showing that the predicted increases in levels of truncated *Htr2c* in brain. Moreover, these molecular and behavioural effects are further associated with evidence of reduced function in specific brain circuits, influenced by 5-HT<sub>2C</sub>R, that control feeding.

Although a change in full-length:truncated *HTR2C* ratio as a consequence of *SNORD115* loss has been



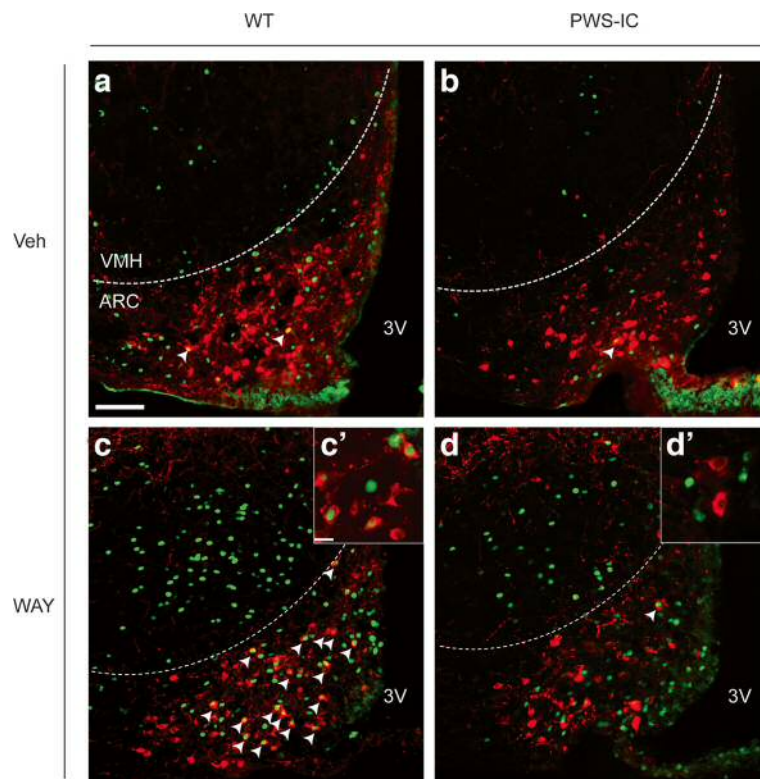
**Fig. 5** Decreased WAY-161503 induced cFOS induction in the ARC of PWS-IC mice. **a–d** Immunohistochemical detection of cFOS immunoreactivity (denoted by black nuclear staining) within the ARC (Bregma  $-1.46$ ) following administration of (**a, b**) vehicle and (**c, d**) 3 mg/kg WAY-161503 to WT and PWS-IC mice. **e, f** Quantification of cFOS induction at two distinct neuroanatomical levels of ARC ( $-1.46$  and  $-1.70$  mm from bregma) and across the nucleus as a whole in (**e**) WT and (**f**) PWS-IC mice following WAY-161503 administration. PWS-IC mice exhibit attenuated ARC cFOS-IR upon WAY-161503 administration. Data presented as Mean  $\pm$  SEM, with statistical comparison performed by Student's *t*-test, \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle controls. Scale bar in (**A**), 100  $\mu$ m and relates to all images. 3 V, third ventricle

predicted for PWS [13], our previous investigation of *Htr2c* pre-mRNA modification in PWS-IC mice at the whole brain level demonstrated an increase of RNA-editing in the absence of a significant effect on proportion of splice variants [18]. However, more neuroanatomically refined analysis of the hypothalamus now reveals concomitant increased levels of the truncated *Htr2c* mRNA with loss of *Snord115* expression in the PWS-IC mice. The truncated variant of 5HT<sub>2C</sub>R can dimerize with the full-length receptor protein, sequestering it to the endoplasmic reticulum [11]. An increase in the abundance of the truncated variant, as occurs in the hypothalamus of PWS-IC mice, would therefore alter the functionality of the serotonin signalling via 5HT<sub>2C</sub>R.

To directly assess the contribution of reduced 5-HT<sub>2C</sub>R signalling on appetite behaviour in this model for PWS, PWS-IC mice were treated with an anorectic dose of a 5-HT<sub>2C</sub>R specific agonist [20]. Our finding that PWS-IC mice exhibited insensitivity to the anorectic

effects of WAY-161503 was correlated with suppressed agonist-induced cFos-IR in ARC POMC neurons of PWS-IC mice. Although we anticipate altered reactivity to WAY-161503 throughout the brain of PWS-IC mice, we focused on ARC neurons because of the well-established link between 5HT<sub>2C</sub>R expression on POMC neurons and satiety and the regulation of body weight [5, 8]. When considered in the context of the basal feeding behaviour of PWS-IC mice [19], these data suggest that *Snord115* deficiency derives less functional 5-HT<sub>2C</sub>R that are incapable of effectuating the anorectic actions of serotonin (via stimulation of downstream POMC signalling) and highlights the independent contribution of this axis to the hyperphagic nature of PWS-IC mice.

A role for abnormal melanocortin signalling in PWS was underlined by ISSH analysis that demonstrated a significant decrease in ARC *Pomc* mRNA, but no differential expression of *Agrp* or *Npy* in PWS-IC mice. This



**Fig. 6** Decreased WAY-161503 induced activation of ARC POMC neurons in PWS-IC mice. Dual immunofluorescence histochemical colocalisation of cFOS (nuclear, green) and POMC (cytoplasmic, red) in the brains of (a, c) vehicle and (b, d) WAY-161503 treated (a, c) WT and (b, d) PWS-IC mice. Inserts in (c, d) represent high magnification images of colocalisation. WAY-161503 administration failed to activate ARC POMC neurons in PWS-IC mice. VMH, ventromedial nucleus of the hypothalamus; 3 V, third ventricle. Scale bar (a), 100  $\mu$ m and relates to (a–d). Scale bar (C'), 25  $\mu$ m and relates to (C' and D')

observation is supported by data from 5-HT<sub>2C</sub>R deficient mice [21] and 5-HT<sub>2C</sub>R-VGV knock-in mice that constitutively express the fully edited isoform of 5HT<sub>2C</sub>R [22], both of which exhibit a reduction in *Pomc* mRNA levels. Together these data suggest that perturbed melanocortinergic signalling may be a functional target for the PWS-IC mutation. Interestingly, elevated *Pomc* expression is observed in neonatal PWS-IC mice and has been implicated in the depressed feeding and early postnatal lethality observed of these mutants [23].

For some time there has been speculation with regards to the importance of 5HT<sub>2C</sub>R mediated appetite in PWS due to the loss of the snoRNA *SNORD115* which negatively regulates the generation of a truncated splice variant of this receptor [13]. To our knowledge this is the first in vivo demonstration that increased levels of truncated *Htr2c* in a mouse model for PWS promotes a disruption of 5-HT<sub>2C</sub> receptor-mediated appetite. Although PWS symptoms observed in very rare cases with an intact *SNORD115* locus discounts this gene as causal of PWS *per se* [24, 25], our data suggest that 5HT<sub>2C</sub>R dysfunction may also contribute to hyperphagia in the majority of cases of PWS where *SNORD115* expression

is lost. However, the PWS-IC model used here also lacks expression of all paternally expressed genes from the PWS imprinting cluster [26]. Consequently, we cannot exclude the involvement the loss of *Necdin* expression in causing alterations in the serotonergic system of the PWS-IC model, as specific knockout of this gene in mice also leads to altered serotonergic neurochemistry [27] although not, as far as we are aware, changes in *Htr2c* splicing. Nevertheless, the data presented here thereby provide new insight into the significance of *Htr2c* pre-mRNA processing to the physiological regulation of appetite and potentially the pathological manifestation of hyperphagia in PWS. Furthermore, these findings have translational relevance for individuals with PWS who may seek to control appetite with another 5-HT<sub>2C</sub>R agonist, namely the new obesity treatment lorcaserin [28].

## Methods

### Animals

PWS-IC animals [29] were bred by transmitting the imprinting centre (IC) mutation paternally (i.e. PWS-IC<sup>+/-</sup>). PWS-IC and WT littermates were housed in



single-sex groups, were subject to a 12 h light/dark cycle, and had *ad libitum* access to standard chow and water, unless stated otherwise.

#### Feeding studies

Daily consumption of a standard (3.68 kcal/g –fat 10%, protein 20%, carbohydrate 70%) and high-fat diet (4.54 kcal/g –fat 45%, protein 20%; carbohydrate 35% - Special Diet Services, Witham, Essex, UK) was assessed over two consecutive 7-day periods. Food consumption was normalised to body weight<sup>0.75</sup> (BW<sup>0.75</sup>).

#### Analysis of *Htr2c* mRNA processing

Whole hypothalamus was macro-dissected using a protocol established in the laboratory [30] and RNA isolated using standard Trizol methods and reverse-transcribed using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). Gene expression was assessed using qPCR, performed with custom designed primers and was analysed using the  $\Delta C_t$  method, normalising to 18 s rRNA as described previously [18]. Ratios of full-length:truncated *Htr2c* were calculated using a modified  $\Delta C_t$  method as follows:

$$Htr2c\text{-full}/Htr2c\text{-trunc}$$

All data were then transformed as usual ( $2^{-\Delta C_t}$ ) before statistical analysis. WT  $n = 10$ , PWS-IC  $n = 8$ .

#### In situ hybridisation histochemistry (ISHH)

Adult brain tissue was collected from animals transcardially perfused with 10% formalin, cryoprotected in 20% sucrose and sectioned at 25  $\mu$ m on a freezing microtome. Tissue was processed for in situ hybridisation as previously described [31]. Radiolabelled riboprobes specific to the mRNA sequences of *Pomc*, *Agrp*, *Npy* and *Bdnf* were used to detect gene expression [7, 32, 33]. Riboprobes were synthesized by PCR using cDNA obtained from normal mouse brain. Recombinant plasmids were linearised by restriction digest and subjected to in vitro transcription with a T7 RNA polymerase in the presence of <sup>35</sup>S-labeled UTP, according to the manufacturer's instructions (Ambion Inc, Austin, Tx). Riboprobes were diluted to 2x10<sup>7</sup> cpm/ml in hybridization solution. Before hybridization, brain sections were fixed in 4% formaldehyde in DEPC-treated PBS and permeabilised by heating in prewarmed sodium citrate buffer (95–100 °C, pH 6.0), before being dehydrated in ascending concentrations of ethanol, and air-dried. Probe solution was applied to each slide and sections were incubated for 12–16 h at 57 °C. After this time slides were incubated in 0.002% RNase A (Qiagen, Valencia, CA), followed by sequential washes in decreasing concentrations of sodium citrate buffer (SSC). The sections were

dehydrated in ascending concentrations of ethanol with 0.3 M ammonium acetate (NH<sub>4</sub>OAc) followed by 100% ethanol. Slides were air-dried and placed in X-ray film cassettes with BMR-2 film (Kodak, UK) for 72 h and developed on an OPTIMAX X-ray film processor. For assessment of gene expression in the autoradiograph films were subjected to densitometry analysis using ImageJ. Integrated mean density values were calculated for each section containing complete ARC.

For *Pomc* and *Npy* analysis,  $n = 4$  for both WT and PWS-IC groups. For *AgRP* and *Bdnf* analysis  $n = 3$  for both groups.

#### WAY-161503 induced cessation of feeding

WAY-161503 hydrochloride (Tocris, Missouri USA) was dissolved in distilled water. The study was conducted as a within-subjects Latin square design, with at least 72 h between treatments. Food was removed at the onset of the dark cycle and the animals fasted for 16 h (water was available *ad libitum*). The following morning male and female WT ( $n = 14$ ) and PWS-IC ( $n = 12$ ) animals were treated with a single injection (sub cutaneous, s.c.) of either vehicle or WAY-161503 (3 and 10 mg/kg) and 15 min later presented with a known weight of mash and allowed to freely consume for 60 min. At the end of the test the remaining food (plus any spillage) was re-weighed. Food consumption was normalised to body weight<sup>0.75</sup> (BW<sup>0.75</sup>).

#### WAY-161503 induced cFOS immunoreactivity (cFOS-IR)

Animals were treated (s.c.) with either 3 mg/kg WAY-161503 or vehicle and brains collected 2 h later after transcardial perfusion with 10% formalin. Brains were cryoprotected in 20% sucrose and sectioned at 25  $\mu$ m on a freezing microtome. Free-floating sections were treated as previously described [4, 7]. Briefly, sections were incubated overnight at room temperature in blocking solution containing rabbit anti-cFOS antibody (Calbiochem; 1:8000), washed, and transferred to blocking solution containing biotinylated donkey anti-rabbit secondary antibody. After rinsing, staining was visualised using following 3,3'-Diaminobenzidine (DAB). For quantification of cFOS-IR, sections containing ARC were assigned a bregma level and the boundaries of the ARC delineated based on neuro-architecture and the Mouse Brain Atlas [34]. ARC cFOS-IR cells falling within the defined regions were counted unilaterally and a bilateral average for the nucleus calculated. For all groups (WT saline, PWS-IC saline, WT WAY-162503, PWS-IC WAY-162503)  $n = 4$ .

#### Dual Immunofluorescence Histochemistry (IHC)

For co-localisation of cFOS-IR and POMC-IR, sections were first treated as detailed above for cFOS-IR and then



processed for POMC immunofluorescence (rabbit anti-POMC – Immunostar, 1:1000; anti-rabbit Alexa Fluor-568 – Molecular Probes, 1:1000). For cFOS/POMC colocalisation sections were imaged on a Zeiss Axioskop 2 microscope and a Zeiss AxioCam HRc digital camera. Chromogenic cFOS signals were photographed in bright-field and fluorescent POMC signal under appropriate excitation wavelength and the images merged in Photoshop CS3 (Adobe Inc).

### Statistics

All data were analysed using SPSS 20 (SPSS, USA). Data were analysed by Student's *t*-test (two-way, unless otherwise indicated) or mixed ANOVA, with between subjects factor of GENOTYPE (PWS-IC vs. WT), and within subject factor DOSE (vehicle, 3 mg/kg and 10 mg/kg WAY-161503). All significance tests were performed at alpha level of 0.05 and where significant interactions were identified in the main ANOVA, *post-hoc* tests using appropriate pair-wise comparisons were performed. For repeated measures analyses, Mauchly's test of sphericity of the covariance matrix was applied.

### Conclusion

Alternate splicing of the serotonin 2C receptor (5-HT<sub>2C</sub>R) is negatively regulated by the *Snord115*, loss of which is seen in most patients with Prader-Willi syndrome (PWS). Given the role of serotonin in the regulation of ingestive behaviour we investigated the pathophysiological implications of *Snord115* deficiency showing that increased levels of a truncated isoform of 5-HT<sub>2C</sub>R in the hypothalamus leads to abnormal 5-HT<sub>2C</sub>R-mediated appetite in a mouse model for PWS. We conclude that loss of *Snord115* expression is physiologically relevant to 5-HT<sub>2C</sub>R mediated appetite which in turn contributes to general hyperphagia in most cases of PWS. These findings also are important for individuals with PWS who may seek to control appetite with the new obesity treatment lorcaserin.

### Additional files

**Additional file 1:** Hypothalamus qPCR analysis. (XLSX 28 kb)

**Additional file 2:** Pomc, AgRP, Npy, Bdnf densitometry. (XLSX 11 kb)

**Additional file 3:** PWS feeding and serotonin pharmacology. (XLS 173 kb)

**Additional file 4:** WAY 0 v 3mg/kg ARC cFos. (XLSX 17 kb)

### Abbreviations

5HT<sub>2C</sub>R: 5-hydroxy-tryptamine 2C receptor; ANOVA: Analysis of variance; ARC: Arcuate nucleus of the hypothalamus; BW: Body weight; DAB: 3,3'-Diaminobenzidine; IHC: Immunohistochemistry; ISSH: In situ hybridisation histology; MCR: Melanocortin receptor; POMC: Pro-opiomelanocortin; PWS: Prader-Willi Syndrome; WT: Wild-type

### Acknowledgements

Not applicable.

### Funding

This work was supported by grants from the Prader-Willi Syndrome Association UK, Wellcome Trust (WT098012; WT081713 to LKH), the Foundation for Prader-Willi Research (to ARI), the Medical Research Council (G0801418) and the Biotechnology and Biological Research Council (BB/J016756/1; to LSW and ARI).

### Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its Additional files 1, 2, 3 and 4.

### Authors' contributions

ASG, LSW, LKH and ARI designed the research. ASG, JRD, LKB, HVF and ARI performed the experiments. ASG and ARI wrote the paper. All authors read and approved the final manuscript.

### Competing interests

A.S.G. is now employed by Pfizer; A.R.I. has received research funding from GW Pharmaceuticals. All other authors declare that they have no competing interests.

### Consent for publication

Not applicable.

### Ethics approval

All procedures were conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986, under the remit of Home Office license number 30/2673. These procedures were also approved by the appropriate ethics committee at Cardiff University.

### Author details

<sup>1</sup>Centre for Integrative Physiology, University of Edinburgh, Edinburgh, UK. <sup>2</sup>Behavioural Genetics Group, MRC Centre for Neuropsychiatric Genetics and Genomics, Neuroscience and Mental Health Research Institute, Schools of Medicine and Psychology, Cardiff University, Cardiff, UK. <sup>3</sup>Department of Pharmacology, University of Cambridge, Cambridge, UK. <sup>4</sup>Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, UK. <sup>5</sup>Present address: Cardiovascular and Metabolic Disease, Pfizer, Cambridge, MA 02139, USA.

Received: 25 May 2016 Accepted: 30 November 2016

Published online: 08 December 2016

### References

- Garfield AS, Heisler LK. Pharmacological targeting of the serotonergic system for the treatment of obesity. *J Physiol*. 2009;587(Pt 1):49–60.
- Lam DD, Garfield AS, Marston OJ, Shaw J, Heisler LK. Brain serotonin system in the coordination of food intake and body weight. *Pharmacol Biochem Behav*. 2010;97(1):84–91.
- Tecott LH, Sun LM, Akana SF, Strack AM, Lowenstein DH, Dallman MF, Julius D. Eating disorder and epilepsy in mice lacking 5-HT<sub>2C</sub> serotonin receptors. *Nature*. 1995;374(6522):542–6.
- Lam DD, Przydzial MJ, Ridley SH, Yeo GS, Rochford JJ, O'Rahilly S, Heisler LK. Serotonin 5-HT<sub>2C</sub> receptor agonist promotes hypophagia via downstream activation of melanocortin 4 receptors. *Endocrinology*. 2008;149(3):1323–8.
- Xu Y, Jones JE, Kohno D, Williams KW, Lee CE, Choi MJ, Anderson JG, Heisler LK, Zigman JM, Lowell BB, et al. 5-HT<sub>2C</sub>Rs expressed by pro-opiomelanocortin neurons regulate energy homeostasis. *Neuron*. 2008;60(4):582–9.
- Xu Y, Jones JE, Lauzon DA, Anderson JG, Balthasar N, Heisler LK, Zinn AR, Lowell BB, Elmquist JK. A serotonin and melanocortin circuit mediates D-fenfluramine anorexia. *J Neurosci*. 2010;30(44):14630–4.
- Heisler LK, Cowley MA, Tecott LH, Fan W, Low MJ, Smart JL, Rubinstein M, Tatro JB, Marcus JN, Holstege H, et al. Activation of central melanocortin pathways by fenfluramine. *Science*. 2002;297(5581):609–11.
- Berglund ED, Liu C, Sohn JW, Liu T, Kim MH, Lee CE, Vianna CR, Williams KW, Xu Y, Elmquist JK. Serotonin 2C receptors in pro-opiomelanocortin neurons regulate energy and glucose homeostasis. *J Clin Invest*. 2013; 123(12):5061–70.
- Canton H, Emeson RB, Barker EL, Backstrom JR, Lu JT, Chang MS, Sanders-Bush E. Identification, molecular cloning, and distribution of a short variant

- of the 5-hydroxytryptamine<sub>2C</sub> receptor produced by alternative splicing. *Mol Pharmacol*. 1996;50(4):799–807.
10. Burns CM, Chu H, Rueter SM, Hutchinson LK, Canton H, Sanders-Bush E, Emeson RB. Regulation of serotonin-<sub>2C</sub> receptor G-protein coupling by RNA editing. *Nature*. 1997;387(6630):303–8.
  11. Martin CB, Ramond F, Farrington DT, Aguiar Jr AS, Chevarin C, Berthiau AS, Caussanel S, Lanfumey L, Herrick-Davis K, Hamon M, et al. RNA splicing and editing modulation of 5-HT<sub>2C</sub> receptor function: relevance to anxiety and aggression in VGV mice. *Mol Psychiatry*. 2013;18(6):656–65.
  12. Zhang Z, Shen M, Gresch PJ, Ghamari-Langroudi M, Rabchevsky AG, Emeson RB, Stamm S. Oligonucleotide-induced alternative splicing of serotonin <sub>2C</sub> receptor reduces food intake. *EMBO Mol Med*. 2016;8(8):878–94.
  13. Kishore S, Stamm S. The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor <sub>2C</sub>. *Science*. 2006;311(5758):230–2.
  14. Vitali P, Basyuk E, Le Meur E, Bertrand E, Muscatelli F, Cavaille J, Huttenhofer A. ADAR2-mediated editing of RNA substrates in the nucleolus is inhibited by C/D small nucleolar RNAs. *J Cell Biol*. 2005;169(5):745–53.
  15. Cavaille J, Buiting K, Kieffmann M, Lalande M, Brannan CI, Horsthemke B, Bachelier JP, Brosius J, Huttenhofer A. Identification of brain-specific and imprinted small nucleolar RNA genes exhibiting an unusual genomic organization. *Proc Natl Acad Sci U S A*. 2000;97(26):14311–6.
  16. Goldstone AP. Prader-Willi syndrome: advances in genetics, pathophysiology and treatment. *Trends Endocrinol Metab*. 2004;15(1):12–20.
  17. Kishore S, Khanna A, Zhang Z, Hui J, Balwiercz PJ, Stefan M, Beach C, Nicholls RD, Zavolan M, Stamm S. The snoRNA MBII-52 (SNORD 115) is processed into smaller RNAs and regulates alternative splicing. *Hum Mol Genet*. 2010;19(7):1153–64.
  18. Doe CM, Relkovic D, Garfield AS, Dalley JW, Theobald DE, Humby T, Wilkinson LS, Isles AR. Loss of the imprinted snoRNA mbii-52 leads to increased 5ht<sub>2c</sub> pre-RNA editing and altered 5HT<sub>2C</sub>-mediated behaviour. *Hum Mol Genet*. 2009;18(12):2140–8.
  19. Davies JR, Humby T, Dwyer DM, Garfield AS, Furby H, Wilkinson LS, Wells T, Isles A. Calorie seeking, but not hedonic response, contributes to hyperphagia in a mouse model for Prader-Willi syndrome. *Eur J Neurosci*. 2015;42(4):2105–13.
  20. Rosenzweig-Lipson S, Zhang J, Mazandarani H, Harrison BL, Sabb A, Sabalski J, Stack G, Welmaker G, Barrett JE, Dunlop J. Antiobesity-like effects of the 5-HT<sub>2C</sub> receptor agonist WAY-161503. *Brain Res*. 2006;1073–1074:240–51.
  21. Nonogaki K, Ohba Y, Sumii M, Oka Y. Serotonin systems upregulate the expression of hypothalamic NUCB2 via 5-HT<sub>2C</sub> receptors and induce anorexia via a leptin-independent pathway in mice. *Biochem Biophys Res Commun*. 2008;372(1):186–90.
  22. Morabito MV, Abbas AI, Hood JL, Kesterson RA, Jacobs MM, Kump DS, Hachey DL, Roth BL, Emeson RB. Mice with altered serotonin <sub>2C</sub> receptor RNA editing display characteristics of Prader-Willi syndrome. *Neurobiol Dis*. 2010;39(2):169–80.
  23. Bittel DC, Kibiryeva N, McNulty SG, Driscoll DJ, Butler MG, White RA. Whole genome microarray analysis of gene expression in an imprinting center deletion mouse model of Prader-Willi syndrome. *Am J Med Genet A*. 2007;143(5):422–9.
  24. de Smith AJ, Purmann C, Walters RG, Ellis RJ, Holder SE, Van Haelst MM, Brady AF, Fairbrother UL, Dattani M, Keogh JM, et al. A deletion of the HBII-85 class of small nucleolar RNAs (snoRNAs) is associated with hyperphagia, obesity and hypogonadism. *Hum Mol Genet*. 2009;18(17):3257–65.
  25. Sahoo T, del Gaudio D, German JR, Shinawi M, Peters SU, Person RE, Garnica A, Cheung SW, Beaudet AL. Prader-Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA cluster. *Nat Genet*. 2008;40(6):719–21.
  26. Relkovic D, Doe CM, Humby T, Johnstone KA, Resnick JL, Holland AJ, Hagan JJ, Wilkinson LS, Isles AR. Behavioural and cognitive abnormalities in an imprinting centre deletion mouse model for Prader-Willi syndrome. *Eur J Neurosci*. 2010;31(1):156–64.
  27. Mercer RE, Kwolek EM, Bischof JM, van Eede M, Henkelman RM, Wevrick R. Regionally reduced brain volume, altered serotonin neurochemistry, and abnormal behavior in mice null for the circadian rhythm output gene *Magel2*. *Am J Med Genet B Neuropsychiatr Genet*. 2009;150B(8):1085–99.
  28. Burke LK, Heisler LK. 5-hydroxytryptamine medications for the treatment of obesity. *J Neuroendocrinol*. 2015;27(6):389–98.
  29. Yang T, Adamson TE, Resnick JL, Leff S, Wevrick R, Francke U, Jenkins NA, Copeland NG, Brannan CI. A mouse model for Prader-Willi syndrome imprinting-centre mutations. *Nat Genet*. 1998;19(1):25–31.
  30. Davies W, Isles A, Smith R, Karunadasa D, Burrmann D, Humby T, Ojarikre O, Biggin C, Skuse D, Burgoyne P, et al. Xlr3b is a new imprinted candidate for X-linked parent-of-origin effects on cognitive function in mice. *Nat Genet*. 2005;37(6):625–9.
  31. Garfield AS, Cowley M, Smith FM, Moorwood K, Stewart-Cox JE, Gilroy K, Baker S, Xia J, Dalley JW, Hurst LD, et al. Distinct physiological and behavioural functions for parental alleles of imprinted *Grb10*. *Nature*. 2011;469(7331):534–8.
  32. Heisler LK, Jobst EE, Sutton GM, Zhou L, Borok E, Thornton-Jones Z, Liu HY, Zigman JM, Balthasar N, Kishi T, et al. Serotonin reciprocally regulates melanocortin neurons to modulate food intake. *Neuron*. 2006;51(2):239–49.
  33. Marston OJ, Garfield AS, Heisler LK. Role of central serotonin and melanocortin systems in the control of energy balance. *Eur J Pharmacol*. 2011;660(1):70–9.
  34. Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates. San Diego: Academic Press; 2001.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at  
[www.biomedcentral.com/submit](http://www.biomedcentral.com/submit)

